Expression and functional analysis of chemokine receptor 7 in canine lymphoma cell lines

Toshitaka KANEI[1)], Munetaka IWATA[1)], Hiroaki KAMISHINA[1)], Takuya MIZUNO[2)] and Sadatoshi MAEDA[1)]*

[1)]The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[2)]Laboratory of Molecular Diagnostics and Therapeutics, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8511, Japan

ABSTRACT. C-C chemokine receptor 7 (CCR7) contributes to cell homing to lymph nodes (LNs). Recent studies reported that CCR7 is also expressed in tumor cells, which correlates with LN metastasis in various cancers. However, the expression of CCR7 in tumor cells is unknown in dogs due to the lack of appropriate antibodies. In the present study, a fusion protein of C-C chemokine ligand 19 (CCL19) was employed as an alternative method to CCR7 antibodies. The fusion CCL19 protein specifically detected CCR7 expressed in canine lymphoma cell lines, which showed active chemotaxis to both canine and mouse ligands. The present study will help further research on the involvement of canine CCR7 in LN metastasis.

KEY WORDS: C-C chemokine ligand 19, C-C chemokine receptor 7, chemotaxis, lymphoma, metastasis

Chemokines are a large family of chemotactic cytokines that induce cell migration. Among the various types of chemokines, C-C chemokine ligand 19 (CCL19), CCL21, and its receptor C-C chemokine receptor 7 (CCR7) play important roles in the functions of cell homing to lymph nodes (LNs). CCL19 and CCL21 are abundantly distributed in LNs, high endothelial venules, and afferent lymphatic vessels in which T-cells and dendritic cells accumulate [1, 5, 17]. Therefore, the CCR7, CCL19, and CCL21 axis forms organized cellular microcompartments in LNs and contributes to the homeostasis of the immune system. Recent studies reported that CCR7 is expressed not only in immune cells, but also in various tumors including lymphoma [22], and breast [15], gastric [14], and lung cancers [19]. Furthermore, the expression of CCR7 in some tumors has been correlated with LN metastasis [16]. In a mouse model, the knockdown of CCR7 in a colorectal cancer cell line decreased LN metastasis [24]. In comparisons with control cells, murine melanoma cells transfected to express CCR7 were found to enhance LN metastasis [20]. The findings of these human and mouse studies implicate CCR7 in LN metastasis in cancer patients.

The involvement of CCR7 in LN metastasis remains unknown in dogs. Previous canine studies employed anti-human CCR7 antibodies [9, 23]; however, their cross-reactivity and specificity to canine CCR7 were not validated. To date, there are no antibodies whose specificity to canine CCR7 has been proven. To detect canine CCR7, the use of a fusion protein combining human CCL19 with human IgG has been reported as an alternative method [8]. However, it is unclear whether the fusion protein binds to canine CCR7 because its specificity was not confirmed. In the present study, we evaluated the specificity of the fusion protein combined with canine CCL19 by validation with canine CCR7 transfectants and T-cells in order to analyze the expression and function of CCR7 in canine lymphoma cell lines which had shown LN metastasis in murine xenograft models [11, 21].

Canine CCL19-human IgG-Fc fusion protein (cCCL19-hIgG-Fc) was prepared as previously described [3, 7]. Concisely, canine full-length CCL19 cDNA (GenBank accession number, AB163919) was inserted into the pCAG-Neo hIgG1-Fc vector (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Human embryonic kidney (HEK) 293A cells (NIBIOHN JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corp.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biosera, Nuaille, France) at 37°C in an atmosphere of air containing 5% CO_2. The pCAG-Neo canine CCL19-hIgG1-Fc plasmid was transfected into HEK293A cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Six hours after transfection, DMEM was exchanged with serum-free medium HE100 (GMEP Inc., Kurume, Japan) including 2 mM L-glutamine. After a further incubation for 6 days, the culture supernatant was collected and purified through ultrafiltration using the Amicon Ultra-15 Centrifugal Filter Unit (Merck,

Darmstadt, Germany). Immunoblotting was conducted to confirm cCCL19-hIgG-Fc in the purified supernatant. In brief, proteins of the purified supernatant were separated by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. One hour after blocking with 5% skim milk, the membrane was incubated with HRP-conjugated goat anti-human IgG-Fc antibody (Thermo Fisher Scientific) at 4°C overnight. Regarding signal development, an enhanced chemiluminescence reaction was conducted with ImmunoStar Zeta (FUJIFILM Wako Pure Chemical Corp.). Images were acquired using a chemiluminometer (ImageQuant LAS 500; Cytiva, Tokyo, Japan).

Canine CCR7 transfectants were prepared using the pMX-IP Retroviral vector (provided by Kitamura, T. at the Institute of Medical Science, The University of Tokyo) containing full-length canine CCR7 cDNA (GenBank accession number, MZ209267). NIH Swiss mouse embryonic fibroblast cells (NIH3T3 cells; provided by the Institute of Development, Aging, and Cancer, Tohoku University) were retrovirally transduced. Transduced NIH3T3 cells were sorted by puromycin selection more than three times. After incubation with blocking buffer containing bovine (Biosera), mouse (Kohjin Bio Inc., Sakado, Japan) and rat (Merck) sera for 30 min, cells were incubated with cCCL19-hIgG-Fc at 4°C for 45 min. Cells were then washed and stained with Allophycocyanin (APC)-conjugated anti-human IgG-Fc (BioLegend, San Diego, CA, USA) as a secondary antibody at 4°C for 30 min. Purified human IgG (FUJIFILM Wako Pure Chemical Corp.) was used as an isotype control. The fluorescence of APC was detected using a flow cytometer (FACSCanto™II; BD Biosciences, Franklin Lakes, NJ, USA) and flow cytometry data were analyzed using flow cytometry software (FlowJo version 10.7.1; Becton Dickinson and Co., Franklin Lakes, NJ, USA).

Peripheral blood mononuclear cells (PBMCs) were collected by density gradient medium (Lymphoprep™; STEMCELL Technologies Inc., Vancouver, Canada) from two 4-year-old female healthy beagle dogs according to the manufacturer's instruction. For the expression analysis of CCR7 in CD8 or CD4 T-cells, PBMCs were stained with a combination of antibodies for CCR7 (cCCL19-hIgG-Fc) and CD8 (FITC-Rat anti Dog CD8 antibody, Clone: YCATE55.9; Bio Rad, Hercules, CA, USA) or CCR7 and CD4 (FITC-Rat anti Dog CD4 antibody, Clone: YKIX302.9; Bio Rad). Appropriate isotype controls were used for CD8 (FITC-Rat IgG$_1$; Bio Rad) and CD4 (FITC-Rat IgG$_{2a}$; Bio Rad), respectively. The proportion of CCR7-positive cells in CD8 or CD4 T-cells was measured using the flow cytometer and software mentioned above. All procedures were approved by the Institutional Animal Care and Use and Clinical Ethics Committees of Gifu University (Accession number, 2020-113).

Three canine lymphoma cell lines, EO-1, CLC, and UL-1, were employed. EO-1 was derived from a dog with cutaneous lymphoma [6]. The origins of CLC and UL-1 were gastrointestinal lymphoma and renal lymphoma, respectively [21]. EO-1 and CLC but not UL-1 had shown LN metastasis in murine xenograft models [11, 21]. Each cell line was cultured in RPMI 1640 medium (FUJIFILM Wako Pure Chemical Corp.) supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine.

The total RNA of each canine lymphoma cell line was extracted using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands). Genomic DNA was removed with a TURBO DNA-free Kit (Applied Biosystems, Foster, CA, USA). Total RNA was reverse transcribed to cDNA using the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Kusatsu, Japan). The transcription of CCR7 was quantified by two-step RT-PCR (Thermal Cycler Dice Real Time System TP800; Takara Bio Inc.) using SYBR Premix Ex Taq™ II (Takara Bio Inc.). PCR primers were as follows: forward (5′-TGGTGGTGGCTCTCCTTGTC-3′) and reverse (5′-AAGTTCCGCACGTCTTTCTTG-3′). Three reference genes, including CG14980, Hypoxanthine Phosphoribosyltransferase 1 (HPRT1), and TBP, were selected by GeNorm and Bestkeeper among nine candidate reference genes (B2M, CG14980, GAPDH, HPRT1, RPL13A, RPL32, RPS18, ACTB, and TBP) [6, 10, 21]. Amplification consisted of a first period of activation (95°C for 10 sec) followed by 40 cycles of a PCR reaction (95°C for 5 sec and 60°C for 30 sec) and dissociation (95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec). Each PCR reaction included samples without reverse transcription, which were not amplified in the present study. The geometric mean of the cycle threshold (Ct) values of the three reference genes were subtracted from the Ct values of CCR7 (ΔCt). All samples were examined in duplicate, and the mean value of ΔCt was calculated. The relative transcription level of CCR7 was calculated by $2^{-\Delta Ct}$, resulting in the evaluation of samples as n-fold differences from the mean value of the three reference genes. The experiment was independently implemented three times. Dunn's test was used to analyze differences in relative CCR7 mRNA levels among the cell lines. A value of $P<0.05$ was considered to be significant. Statistical analyses were performed using the JMP 13.2.0 program (SAS Institute, Gary, NC, USA).

The detection of CCR7 in EO-1, CLC, and UL-1 by flow cytometry using cCCL19-hIgG-Fc was conducted in the same manner as canine CCR7 transfectants and T-cells described above.

A chemotaxis assay was performed in a 24-transwell plate with a 5-µm pore insert (Corning Inc., Corning, NY, USA). Canine and mouse recombinant CCL19 (Kingfisher Biotech Inc., Saint Paul, MN, USA) (Prospec, Ness-Ziona, Israel) were diluted in RPMI 1640 from 0.1 to 500 nmol/l, and 600 µl of the diluted solution was placed in the lower chamber. EO-1 and UL-1 were resuspended in RPMI 1640 at 1×10^7 cells/ml, and 100 µl of each cell suspension was added to the upper chamber. After an incubation for 3 hr at 37°C under 5% CO$_2$, the number of migrated cells in the lower chamber was counted. The assay was performed in duplicate.

Immunoblotting detected a single band near 37 kDa corresponding to the expected molecular weight of canine CCL19 combined with the human IgG-Fc region in the purified supernatant (Fig. 1a). A flow cytometric analysis showed that cCCL19-hIgG-Fc bound to NIH3T3 cells transfected with canine CCR7, but not to non-transfectants (Fig. 1b). The proportion of CCR7-positive CD8 T-cells was 51.9% and 30.6% in dog 1 and 2, respectively. While, the ratio of CCR7-positive CD4 T-cells was 77.3% and 82.1% in dog 1 and 2, respectively (Fig. 2). The qRT-PCR analysis showed that CCR7 mRNA expression levels were significantly higher in EO-1 than in UL-1 (Fig. 3a). The flow cytometric analysis also revealed that positive staining was moderate in EO-1 and dim in CLC, but almost negative in UL-1 (Fig. 3b). In the chemotaxis assay, the number of migrated EO-1 cells increased in proportion to
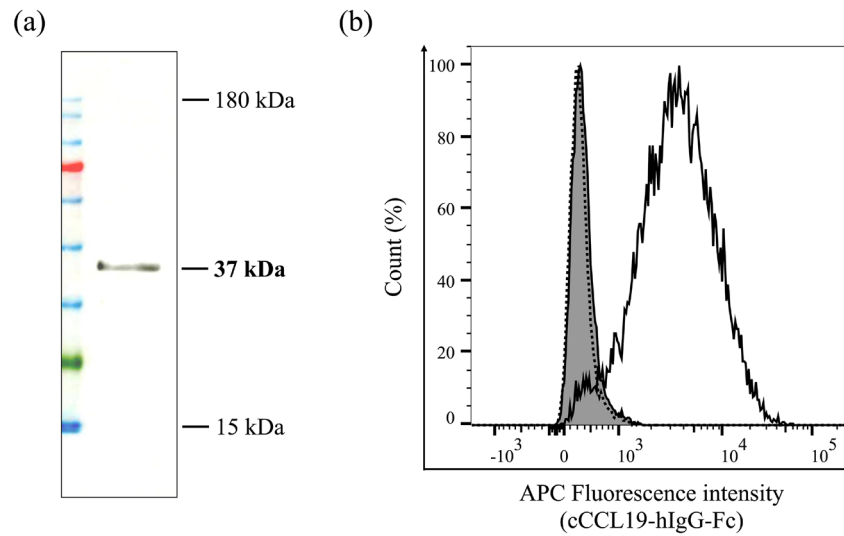
(a)   (b)

Fig. 1. Immunoblotting and flow cytometric analysis of canine C-C chemokine ligand 19-human IgG-Fc (cCCL19-hIgG-Fc). (**a**) Immunoblotting using an anti-human IgG-Fc antibody in the purified supernatant. (**b**) The flow cytometric analysis showed that cCCL19-hIgG-Fc bound to canine C-C chemokine receptor 7 (CCR7)-transfectants (white area), but not to non-transfectants (dotted line). The grey area represents the isotype control.
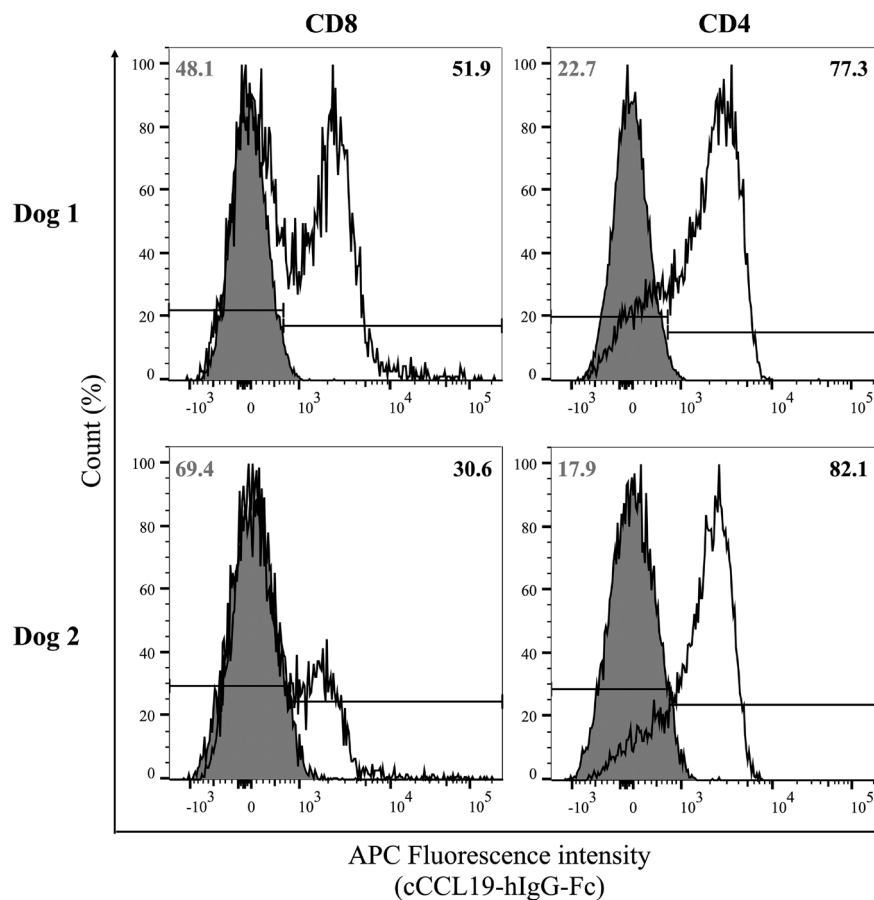

Fig. 2. Expression analysis of C-C chemokine receptor 7 (CCR7) in CD8 and CD4 T-cells from two healthy dogs. The histogram shows staining intensities by the isotype control (grey area) and canine C-C chemokine ligand 19-human IgG-Fc (cCCL19-hIgG-Fc) (white area). The proportion of CCR7-positive and negative cells is shown in black and grey letters, respectively.
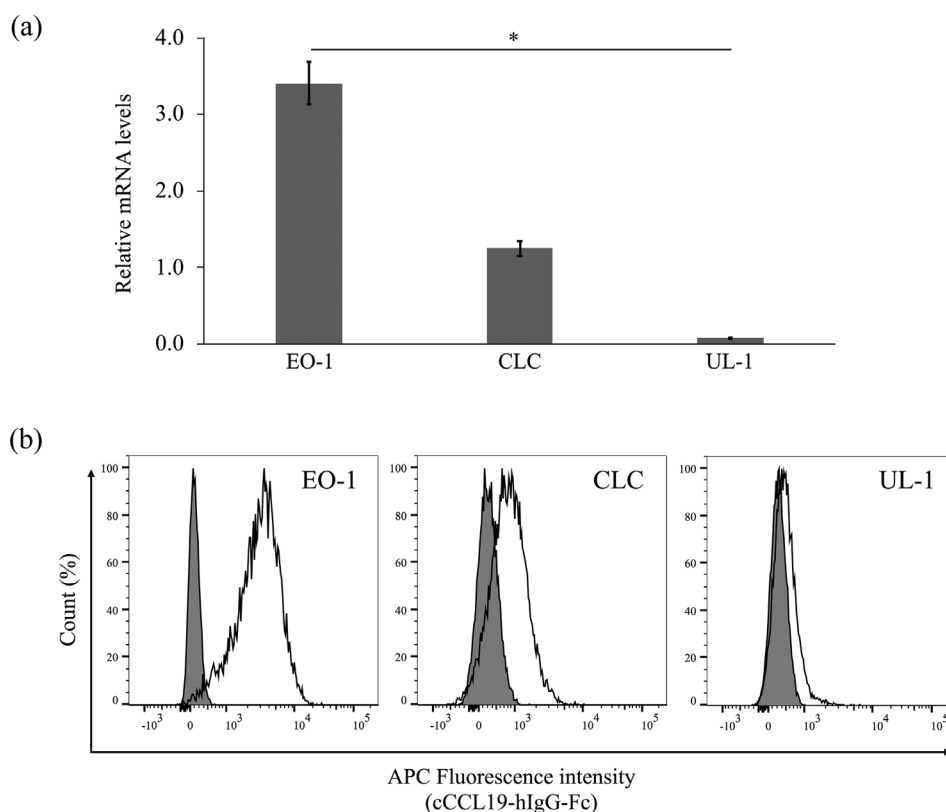
(a)

(b)

APC Fluorescence intensity
(cCCL19-hIgG-Fc)

Fig. 3. Expression analysis of C-C chemokine receptor 7 (CCR7) in canine lymphoma cell lines (EO-1, CLC, and UL-1). (**a**) The expression of canine CCR7 mRNA was shown as a relative quantity. Results are shown as the means ± standard deviation of three independent experiments. *$P<0.05$ by Dunn's test. (**b**) The histogram shows staining intensities by the isotype control (grey area) and canine C-C chemokine ligand 19-human IgG-Fc (cCCL19-hIgG-Fc) (white area).
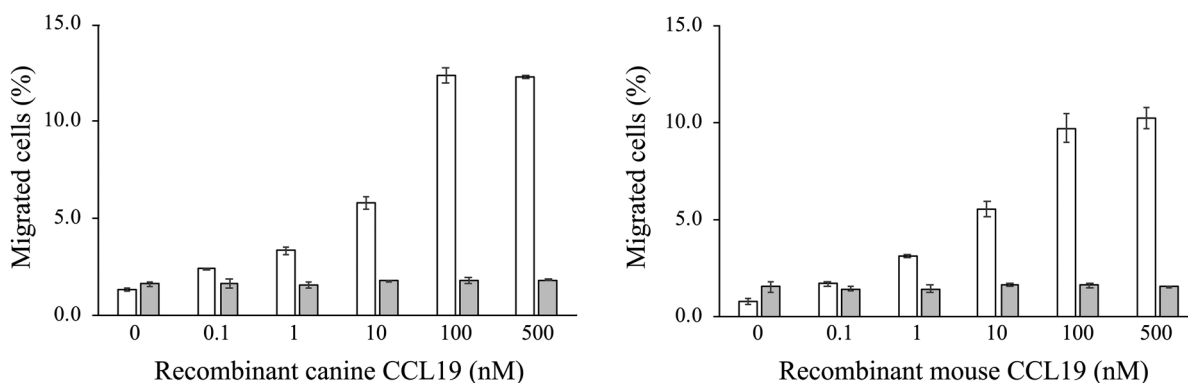

Fig. 4. Chemotaxis assay using canine and mouse recombinant C-C chemokine ligand 19 (CCL19). White bars represent EO-1, and grey bars represent UL-1. Results are shown as the means ± standard deviation of two independent experiments.

the concentration of both canine and mouse CCL19. In contrast, UL-1 showed no increase in the number of migrated cells (Fig. 4).

In canine studies, anti-human antibodies have been used to detect chemokine receptors, such as CCR4 and CCR9 [12, 13]. We examined six antibodies for human or mouse CCR7; however, none were cross-reactive to canine CCR7 (Supplementary Data). These results prompted us to focus on the receptor-ligand binding assay using the fusion protein cCCL19-hIgG-Fc. Since the specificity of CCL19-hIgG to canine CCR7 was previously reported to be unclear [8], we initially validated the specificity by using the canine CCR7 transfectants. The results obtained demonstrated that cCCL19-hIgG-Fc specifically bound to canine CCR7. We also indicated that the cCCL19-hIgG-Fc was able to detect CCR7 in T-cells from healthy dogs. The expression of CCR7 in CD8 T-cells in the present study varied between the dogs the same as in the previous report [8], while the proportion of CCR7-positive CD4 T-cells was higher, which may be attributed to the high affinity between canine receptor and canine ligand not human.

In clinical cases, human gastric carcinoma demonstrated that CCR7-positive tumor cells were detected in 66% of patients, which correlated with LN metastasis and lymphatic invasion [14]. In esophageal squamous cell carcinoma in humans, the CCR7 expression in patients with LN metastasis was significantly higher than those without LN metastasis [4]. These results suggest that CCR7 expression in tumor tissues may be a potential biomarker to predict LN metastasis. In dogs, CCR7 expression has not been evaluated in tumor tissues. Canine CCL19-hIgG-Fc validated in the present study applies to the detection of canine CCR7 in tumor tissues; however, the detection is limited for living cells. For expression analysis in canine tumors, therefore, the method for living cell isolation from clinical samples should be developed and standardized in veterinary clinics.

The results of transcription and expression analyses showed that the expression of CCR7 varied among canine lymphoma cell lines. We employed EO-1 as the cell line showing the highest expression levels of CCR7 and UL-1 as that with the lowest for the chemotaxis assay to evaluate the function of canine CCR7. The results of the assay indicated the active chemotaxis of EO-1 not only to canine but also to mouse recombinant CCL19, which ensures mouse models xenografted with canine cell lines to evaluate the roles of canine CCR7 in tumor cell dynamics. Previous studies reported the metastasis of regional LNs in 4/4 and 3/4 mice xenografted with EO-1 or CLC, respectively [11, 21], but not in mice xenografted with UL-1 [21]. Since the major function of CCR7 is homing to LNs, the metastasis of regional LNs in these mouse models appeared to be related to the expression of CCR7 in xenografted tumor cells. Further studies including the knockdown or knockout of CCR7 in EO-1 and CLC are required to clarify the involvement of canine CCR7 in metastasis.

In humans, CCR7 has been targeted for a novel cancer treatment. A monoclonal CCR7 antibody has already demonstrated successful efficacy in a mantle lymphoma model [18] and T-cell leukemia xenograft model [2]. Therefore, research on CCR7 is of great significance for elucidating the mechanisms underlying tumor metastasis and the development of novel treatments. In view of the high sequence homology of CCR7 between canines and humans, the strategy targeting CCR7 may be applicable to cancers in dogs. Canine CCR7 transfectants allow for the manufacture of a monoclonal antibody to canine CCR7, which may lead to the development of novel treatments.

In conclusion, the present study revealed that canine tumor cell lines expressed functional CCR7. The cCCL19-hIgG-Fc fusion protein employed here is specific to detect canine CCR7, providing further application for prospective canine studies.

CONFLICT OF INTEREST. The authors declare that they have no conflicts of interest related to the subject materials discussed in this article.

REFERENCES

1. Bromley, S. K., Thomas, S. Y. and Luster, A. D. 2005. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat. Immunol.* **6**: 895–901. [Medline] [CrossRef]
2. Cuesta-Mateos, C., Fuentes, P., Schrader, A., Juárez-Sánchez, R., Loscertales, J., Mateu-Albero, T., Vega-Piris, L., Espartero-Santos, M., Marcos-Jimenez, A., Sánchez-López, B. A., Pérez-García, Y., Jungherz, D., Oberbeck, S., Wahnschaffe, L., Kreutzman, A., Andersson, E. I., Mustjoki, S., Faber, E., Urzainqui, A., Fresno, M., Stamatakis, K., Alfranca, A., Terrón, F., Herling, M., Toribio, M. L. and Muñoz-Calleja, C. 2020. CCR7 as a novel therapeutic target in t-cell PROLYMPHOCYTIC leukemia. *Biomark. Res.* **8**: 54. [Medline] [CrossRef]
3. Debes, G. F., Arnold, C. N., Young, A. J., Krautwald, S., Lipp, M., Hay, J. B. and Butcher, E. C. 2005. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat. Immunol.* **6**: 889–894. [Medline] [CrossRef]
4. Ding, Y., Shimada, Y., Maeda, M., Kawabe, A., Kaganoi, J., Komoto, I., Hashimoto, Y., Miyake, M., Hashida, H. and Imamura, M. 2003. Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clin. Cancer Res.* **9**: 3406–3412. [Medline]
5. Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E. and Lipp, M. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**: 23–33. [Medline] [CrossRef]
6. Hara, K., Iio, A., Asahina, R., Takahashi, M., Mori, T., Nishida, H., Kamishina, H., Sakai, H., Kitoh, K., Mizuno, T., Tsujimoto, H. and Maeda, S. 2017. Characterization of a novel canine T-cell line established from a dog with cutaneous T-cell lymphoma. *J. Dermatol. Sci.* **88**: 254–256. [Medline] [CrossRef]
7. Hargreaves, D. C., Hyman, P. L., Lu, T. T., Ngo, V. N., Bidgol, A., Suzuki, G., Zou, Y. R., Littman, D. R. and Cyster, J. G. 2001. A coordinated change in chemokine responsiveness guides plasma cell movements. *J. Exp. Med.* **194**: 45–56. [Medline] [CrossRef]
8. Hartley, A. N. and Tarleton, R. L. 2015. Chemokine receptor 7 (CCR7)-expression and IFNγ production define vaccine-specific canine T-cell subsets. *Vet. Immunol. Immunopathol.* **164**: 127–136. [Medline] [CrossRef]
9. Hillman, L. A., Garrett, L. D., de Lorimier, L. P., Charney, S. C., Borst, L. B. and Fan, T. M. 2010. Biological behavior of oral and perioral mast cell tumors in dogs: 44 cases (1996–2006). *J. Am. Vet. Med. Assoc.* **237**: 936–942. [Medline] [CrossRef]
10. Iio, A., Motohashi, T., Kunisada, T., Yasuhira, Y., Kamishina, H. and Maeda, S. 2014. Preferential gene transcription of T helper 2 cytokines in peripheral CCR4+ CD4+ lymphocytes in dogs. *Vet. Dermatol.* **25**: 199–e50. [Medline] [CrossRef]
11. Ikeuchi, M., Asahina, R., Nishida, H., Kamishina, H., Kitoh, K., Sakai, H. and Maeda, S. 2018. Phenotypic analysis of mice xenografted with canine epitheliotropic cutaneous T-cell lymphoma cells. *Vet. Dermatol.* **29**: 517–e172. [Medline] [CrossRef]
12. Maeda, S., Okayama, T., Omori, K., Masuda, K., Sakaguchi, M., Ohno, K. and Tsujimoto, H. 2002. Expression of CC chemokine receptor 4 (CCR4) mRNA in canine atopic skin lesion. *Vet. Immunol. Immunopathol.* **90**: 145–154. [Medline] [CrossRef]
13. Maeda, S., Ohno, K., Tsukamoto, A., Nakashima, K., Fukushima, K., Goto-Koshino, Y., Fujino, Y. and Tsujimoto, H. 2012. Molecular cloning and expression analysis of the canine chemokine receptor CCR9. *Vet. Immunol. Immunopathol.* **145**: 534–539. [Medline] [CrossRef]
14. Mashino, K., Sadanaga, N., Yamaguchi, H., Tanaka, F., Ohta, M., Shibuta, K., Inoue, H. and Mori, M. 2002. Expression of chemokine receptor

CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res.* **62**: 2937–2941. [Medline]

15. Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verástegui, E. and Zlotnik, A. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**: 50–56. [Medline] [CrossRef]

16. Rizeq, B. and Malki, M. I. 2020. The Role of CCL21/CCR7 chemokine axis in breast cancer progression. *Cancers (Basel)* **12**: 12. [Medline] [CrossRef]

17. Sallusto, F. and Baggiolini, M. 2008. Chemokines and leukocyte traffic. *Nat. Immunol.* **9**: 949–952. [Medline] [CrossRef]

18. Somovilla-Crespo, B., Alfonso-Pérez, M., Cuesta-Mateos, C., Carballo-de Dios, C., Beltrán, A. E., Terrón, F., Pérez-Villar, J. J., Gamallo-Amat, C., Pérez-Chacón, G., Fernández-Ruiz, E., Zapata, J. M. and Muñoz-Calleja, C. 2013. Anti-CCR7 therapy exerts a potent anti-tumor activity in a xenograft model of human mantle cell lymphoma. *J. Hematol. Oncol.* **6**: 89. [Medline] [CrossRef]

19. Sun, L., Zhang, Q., Li, Y., Tang, N. and Qiu, X. 2015. CCL21/CCR7 up-regulate vascular endothelial growth factor-D expression via ERK pathway in human non-small cell lung cancer cells. *Int. J. Clin. Exp. Pathol.* **8**: 15729–15738. [Medline]

20. Takekoshi, T., Fang, L., Paragh, G. and Hwang, S. T. 2012. CCR7-expressing B16 melanoma cells downregulate interferon-γ-mediated inflammation and increase lymphangiogenesis in the tumor microenvironment. *Oncogenesis* **1**: e9. [Medline] [CrossRef]

21. Umeki, S., Ema, Y., Suzuki, R., Kubo, M., Hayashi, T., Okamura, Y., Yamazaki, J., Tsujimoto, H., Tani, K., Hiraoka, H., Okuda, M. and Mizuno, T. 2013. Establishment of five canine lymphoma cell lines and tumor formation in a xenotransplantation model. *J. Vet. Med. Sci.* **75**: 467–474. [Medline] [CrossRef]

22. Yang, J., Wang, S., Zhao, G. and Sun, B. 2011. Effect of chemokine receptors CCR7 on disseminated behavior of human T cell lymphoma: clinical and experimental study. *J. Exp. Clin. Cancer Res.* **30**: 51. [Medline] [CrossRef]

23. Yarnall, B. W., Chamberlain, C. S., Hao, Z. and Muir, P. 2019. Proinflammatory polarization of stifle synovial macrophages in dogs with cruciate ligament rupture. *Vet. Surg.* **48**: 1005–1012. [Medline] [CrossRef]

24. Yu, S., Duan, J., Zhou, Z., Pang, Q., Wuyang, J., Liu, T., He, X., Xinfa, L. and Chen, Y. 2008. A critical role of CCR7 in invasiveness and metastasis of SW620 colon cancer cell in vitro and in vivo. *Cancer Biol. Ther.* **7**: 1037–1043. [Medline] [CrossRef]